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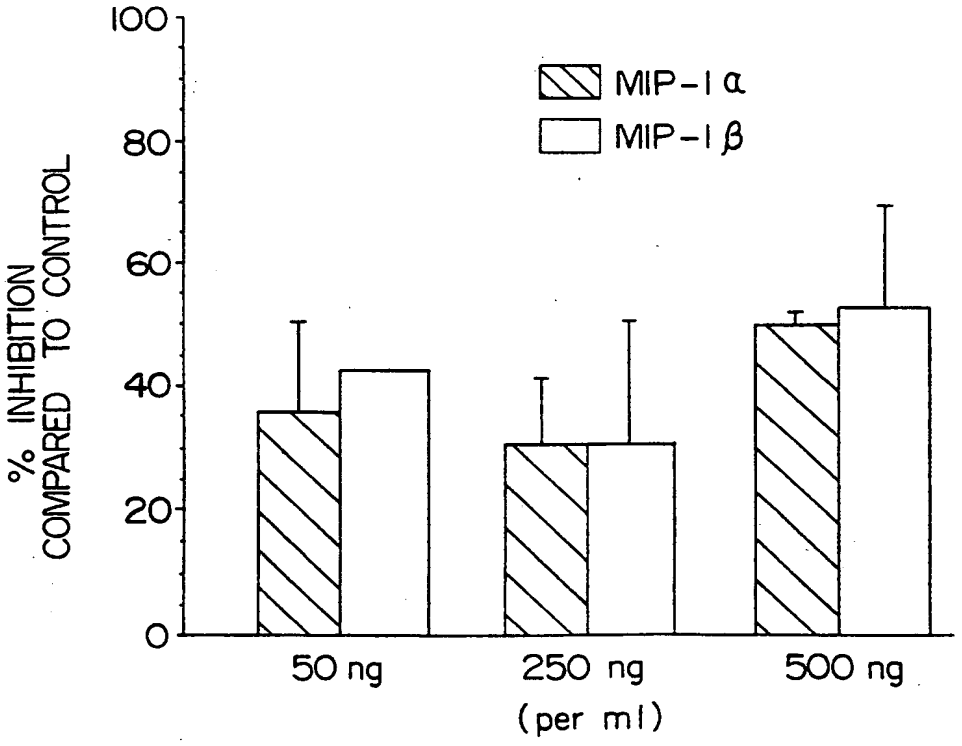
(54) Title: SUPPRESSION OF MEGAKARYOCYTOPOIESIS BY MACROPHAGE INFLAMMATORY PROTEINS



(57) Abstract

Macrophage inflammatory protein-1 or -2, or analog thereof, is administered to a mammal to achieve therapeutic reduction of the number of circulating platelets. The proteins are useful in treating essential thrombocythemia and reactive thrombocytosis.

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SUPPRESSION OF MEGAKARYOCYTOPOIESIS BY MACROPHAGE INFLAMMATORY PROTEINS

Field of the Invention

The invention relates to the inhibition of megakaryocytopoiesis.

Reference to Government Grant

The invention was made with government support under grant CA 36896 awarded by the National Institutes of Health. The government has certain rights in the invention.

Background of the Invention

Pluripotent hematopoietic stem cells are activated in the bone marrow to proliferate and differentiate into mature megakaryocytes, each of which is capable of releasing up to several thousand functional platelets in response to biological demand. Development of the stem cell proceeds by stages broadly corresponding to proliferation of progenitor cells, and differentiation of late progenitor and early precursor cells into mature megakaryocytes. Although regulation of this developmental process (megakaryocytopoiesis) is of substantial clinical interest for its potential application to disorders characterized by abnormal platelet production, endogenous factors responsible for stimulating or inhibiting proliferation and differentiation

of megakaryocyte progenitor/precursor cells have not been thoroughly elaborated.

Thrombocytosis is a condition marked by the absolute increase in the number of circulating platelets. In some cases the elevation is acute and transient; in others it is chronic and persistent. The term "reactive thrombocytosis" has been commonly applied to define the concept that these patients have increased circulating platelet numbers in response to some underlying disease. This is in contrast to the condition where an autonomous drive to platelet production exists, commonly termed "thrombocythemia".

Reactive thrombocytosis may appear and persist as a result of chronic blood loss with iron deficiency, chronic inflammatory disease, chronic infectious disease, cancer and hemolytic anemia.

Primary thrombocythemia, also known as essential thrombocythemia, is an autonomous clonal proliferation of a pluripotent hematopoietic stem cell that results in an absolute increase in the number of circulating platelets. It shares several clinical features with other myeloproliferative disorders, most notably frequent bleeding and thrombotic lesions that represent major causes of morbidity and mortality.

Inhibitory factors capable of clinically significant megakaryocyte suppression have not been well-characterized. For example, both immunocytes and transforming growth factor-β (TGF-β) have been studied as potential inhibitors of megakaryocytopoiesis, with inconclusive results (see, e.g., Blood 67, 479-483 and Blood 68, 619-626, (1986)). Additionally, autoregulation via negative feedback mechanisms involving megakaryocyte products, including platelet-secreted 12-17kD glycoprotein, has been reported (J. Cell Physiol. 130, 361-368, (1987)). Platelet factor 4 and a synthetic C-terminal peptide have been shown to be capable of inhibiting megakaryocytopoiesis (Gewirtz et al., J.

Clin Invest. 83, 1477-1486 (1989)). It has also been suggested that interferon-α and interferon-γ may have a role in regulating megakaryocyte colony formation (Ganser et al., Blood 70, 1173-1179 (1987); Chott et al., Br. J. Haematol. 74, 10-16 (1990)). While interferon-α has been used to lower platelet counts in patients with primary thrombocythemia and thrombocytosis associated with other types of malignant lesions, only approximately about 50% of patients achieve a stable state of remission. Moreover, on cessation of interferon therapy, recurrence of clinical and laboratory findings is usual (Gisslinger et al., Lancet 1, 634-637 (1989)).

While the potential utility of negative autocrine regulators or other megakaryocytopoiesis inhibitors in the clinical treatment of disorders characterized by excessively high platelet counts is apparent, none of the heretofore postulated inhibitors has so far proved useful in such applications.

Cytoreducive chemotherapeutic agents such as alkylating agents, radiophosporous and antimetabolites have been used to reduce platelet numbers. Most have leukemogenic potential. Their use has largely been abandoned in favor of hydroxyurea. However, hydroxyurea should at best be considered an agent with uncertain carcinogenic potential because at least one case of primary thrombocythemia conversion to acute leukemia has been linked to hydroxyurea therapy (Anker-Lugtenberg et al., Am. J. Hematol. 33:152 (1990)).

Anagrelide, a member of the imidazo(2,1-b)quinazolin-2-one series, is an investigational drug which has been recently proposed for the treatment of thrombocytosis. Anagrelide has been shown to be capable of controlling platelet counts in most patients suffering from essential thrombocythemia as a consequence of an underlying myeloproliferative disorder. Suppression of platelet counts by anagralide appears to be selective

relative to changes in white blood cell count and hemoglobin. However, the drug's potent effect on inhibiting platelet activation requires further study.

Macrophage inflammatory protein-1 (MIP-1) is a heparin-binding protein secreted by macrophages in response to lipopolysaccharide stimulation. MIP-1 is a major secretion product from stimulated macrophages, comprising about 2% of proteins secreted by endotoxin-stimulated cells. MIP-1 causes a local inflammatory response in mice and induces superoxide production in human neutrophils in vitro. It is also mildly chemokinetic for human neutrophils. MIP-1 is composed of two distinct peptides, MIP-1α and MIP-1β.

Macrophage inflammatory protein-2 (MIP-2), is another heparin-binding protein secreted by lipopolysaccharide-stimulated macrophages. It comprises about 0.5% of the proteins secreted by stimulated macrophages. Like MIP-1, MIP-2 has been shown to elicit a local inflammatory response when injected subcutaneously into mice. It has potent chemotactic activity for human polymorphonucleocytes. Also like MIP-1, MIP-2 is composed of two distinct peptides, MIP-2α and MIP-2β.

The activity of MIP-1 and MIP-2 in inhibiting megakaryocytopoiesis was unknown prior to the invention hereinafter described.

Summary of the Invention

A method for suppressing megakaryocytopoiesis in a mammal is provided, which results in the reduction of the number of circulating platelets in the bloodstream of that mammal. An effective amount of macrophage inflammatory protein-1 (MIP-1), macrophage inflammatory protein-2, or analog of either, is administered to effect such platelet reduction. The invention is particularly useful in the treatment of disorders characterized by an excessively high platelet count.

MIP suppresses megakaryocyte maturation (i.e., differentiation).

By "analog" with respect to MIP-1 or MIP-2 is meant a modified polypeptide having an amino acid sequence substantially the same as that of either MIP-1α or MIP-1β, or either MIP-2α or MIP-2β, respectively, in which one or more amino acids have been deleted or substituted, or in which one or more amino acids have been inserted; which modified polypeptide retains the property of inhibiting megakaryocytopoiesis.

Description of the Figures

Figure 1 is a graph of the effect of murine MIP-1α and MIP-1β on megakaryocyte colony formation at concentrations of 50, 250 and 500 ng/ml. The data are expressed as a percentage of inhibition compared to a control culture (no MIP).

Figure 2 is a graph of the effect of human MIP-2α and MIP-2β on megakaryocyte colony formation at concentrations of 50, 250 and 500 ng/ml. The data are again expressed as a percentage of inhibition compared to a control (no MIP).

Detailed Description of the Invention.

According to the invention, MIP-1α, -1β, -2α or -2β (collectively "MIP"), or analog thereof, is employed to inhibit megakaryocytopoiesis to effect in vivo reduction of platelet numbers. Sufficient MIP is given, preferably by intravenous administration, to decrease the number of circulating platelets. Depending upon the route of administration and idiosyncratic factors, most particularly the individual platelet count and the rate of MIP clearance, the average dosage may be as little as about 2.5 mg per day, up to several grams per day, for a human subject.

Murine MIP-1α and MIP-1β have been isolated and cloned. See: Sherry et al., J. Exp. Med. 168,

2251-2259 (1988) (isolation of murine MIP-1 from endotoxin-stimulated RAW 264.7 cells (ATCC), followed by resolution of α and β types by SDS-hydroxylapatite chromatography, construction of a cDNA library and sequencing of the relevant cDNA). More recently, the genes coding for human MIP-1α and MIP-1β have been identified and the relevant cDNA and predicted amino acid sequences determined (Zipfel et al., J. Immunol. 142, 1582-1590 (1989); Irving et al., Nucleic Acids Res. 18, 3261-3270 (1990), both incorporated herein by reference). The human MIP-1α gene is designated "464" by Irving et al. The nucleotide sequence of the relevant cDNA and the predicted amino acid sequence are indicated in Fig. 1 thereof, and in Fig. 3 of Zipfel et al under the prior name "pAT 464" for the same gene. The cDNA and amino acid sequences for MIP-1α is also available from the EMBL/GenBank Data Library under accession number X52149.

The human MIP-1β gene has been designated "pAT 744" and "744.1" by the same investigators. The nucleotide sequence of the MIP-1β cDNA, and the predicted amino acid sequence, are indicated in Fig. 4 of Zipfel et al under the name pAT 744.

The amino acid sequences of human MIP-1α and MIP-1β are reproduced herein as SEQ ID NO:1 and SEQ ID NO:2, respectively.

Murine MIP-2 has previously been isolated and cloned. See: Wolpe et al., Proc. Natl. Acad. Sci. USA 86, 612-616 (1989) (isolation of murine MAP-2 from endotoxin-stimulated RAW 264.7 cells (ATCC)); Tekamp-Olson et al., J. Exp.Med. 172, 911-919 (1990) (cloning of murine MIP-2). The latter investigators utilized a fragment of murine MIP-2 cDNA encoding most of the mature murine MIP-2 protein to probe a cDNA library prepared from poly(A)$^+$ RNA of the human monocytic-like cell line U937 (ATCC). The nucleotide sequence and predicted amino acid sequences of human MIP-2α and

human MIP-2β have thus been reported (Id.) The cDNA and amino acid sequences are also available from the EMBL/GenBank Data Library under accession numbers X53799 (MIP-2α) and X53800 (MIP-2β). The disclosure of Tekamp-Olson et al. is incorporated herein by reference.

The amino acid sequences of human MIP-2α and MIP-2β are reproduced herein as SEQ ID NO:3 and SEQ ID NO:4, respectively.

MIP-1 and -2, and megakaryocytopoiesis-inhibiting analogs thereof, may be chemically synthesized by conventional solid phase synthetic techniques initially described by Merrifield, in J. Am. Chem. Soc. 15, 2149-2154 (1963). Other peptide synthesis techniques may be found, for example, in M. Bodanszky et al., Peptide Synthesis, John Wiley & Sons, 2d Ed. (1976) as well as in other reference works known to those skilled in the art. A summary of peptide synthesis techniques may be found in J. Stuart and J.D. Young, Solid Phase Peptide Synthesis, Pierce Chemical Company, Rockford, IL (1984). The synthesis of peptides by solution methods may also be used, as described in The Proteins, vol-II, 3d Ed., Neurath, H. et al., Eds., p. 105-237, Academic Press, New York, NY (1976). Appropriate protective groups for use in such syntheses will be found in the above texts as well as in J. F. W. McOmie, Protective Groups in Organic Chemistry, Plenum Press, New York, NY (1973).

In general, these synthetic methods involve the sequential addition of one or more amino acid residues or suitably protected amino acid residues to a growing peptide chain. Normally, either the amino or carboxyl group of the first amino acid residue is protected by a suitable, selectively-removable protecting group. A different, selectively-removable protecting group is utilized for amino acids containing a reactive side group, such as lysine.

Since the MIP amino acid sequences are known, the proteins may of course also be prepared by recombinant DNA techniques well-known to those skilled in the art. Moreover, analogs involving the substitution, deletion or insertion of one or more amino acids may similarly be prepared by such recombinant techniques, or by solid or liquid phase peptide syntheses, as described above.

It is contemplated, based upon the available MIP amino acid sequences, that MIP analogs may be prepared and effectively screened for ability to inhibit megakaryocytopoiesis according to the megakaryocyte assay hereinafter described. In particular, it is contemplated that conservative amino acid changes may be made which do not alter the biological function of the peptide.

For instance, one polar amino acid, such as glycine, may be substituted for another polar amino acid; or one acidic amino acid, such as aspartic acid may be substituted for another acidic amino acid, such as glutamic acid; or a basic amino acid, such as lysine, arginine or histidine may be substituted for another basic amino acid; or a non-polar amino acid, such as alanine, leucine or isoleucine may be substituted for another non-polar amino acid.

The degree of homology between the MIP analog and the corresponding native MIP amino acid sequence is preferably at least 80%, more preferably at least 90%, most preferably at least 95%.

MIP or MIP analogs are contemplated for use according to the invention in lowering blood levels of circulating platelets as deemed clinically advantageous, and for use in reducing the ability of these platelets to support blood clot formation. Pathological vascular reactions associated with excessively high platelet counts include stroke, pulmonary emboli, and related thromboembolic complications. A predisposing factor of these potentially fatal complications, high

subcutaneous injection, with intravenous injection being preferred.

For intravenous administration, the peptide may be dissolved in an appropriate intravenous delivery vehicle containing physiologically compatible substances such as NaCl, glycine and the like, having a buffered pH compatible with physiologic conditions. Such intravenous delivery vehicles are known to those skilled in the art.

MIP or analog thereof may be operatively linked to a pharmaceutically acceptable carrier molecule to form a megakaryocytopoiesis-inhibiting complex. By "operatively linked" is meant any form of chemical or physical association or bond, including, but not limited to non-covalent complex formation, covalent bonding (including but not limited to covalent bonding by one or more cross-linking agents), and the like, which does not substantially interfere with the megakaryocytopoiesis-inhibiting activity of MIP.

Typically, the carrier molecule will comprise a protein, such as albumin, to improve the delivery of MIP and/or prolonging the half-life of MIP in the body. Non-protein carriers include, for example polyethylene glycol. Other cytokines, *e.g.*, interleukin-2, have been conjugated to polyethylene glycol to improve circulatory half-life and activity. See, for example, Mattussen et al.; Int. J. Cancer 51, 812-817 (1992); Meyers et al., Clin. Pharmacol. Ther. 49, 307-313 (1991); and Knauf et al., J. Biol. Chem. 263, 15064-15070 (1988).

Techniques for protein conjugation through activated functional groups are particularly applicable. For a review of such techniques, see Aurameas et al., Scan. J. Immunol. 8, Supp. 7, 7-23 (1978). Also see U. S. Patents 4,493,795 and 4,671,958. A wide range of homobifunctional and heterobifunctional cross-linking agents for covalently linking proteins are well known to those skilled in the art. The following is a

partial list of such agents, as listed international patent application WO 90/14102 (1990), p. 29-31:

Homobifuncitonal cross-linking reagents for linking the macrophage inflamatory protein to a carrier protein include, for example, disuccinimidyl tartrate, disuccinimidyl suberate, ethylene glycolbis (succinimidyl succinate), 1,5-difluoro-2-dinitrobenzene ("DFDNB"), 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene ("DIDS") and bismaleimidohexane ("BMH").

Alternatively, heterobifunctional cross-linking reagents may be employed. Such agents include, for example, N-succinimidyl-2-(2-pyridyldithio)proprinate ("SPDP"), sulfosuccinimidyl-2-(pazidosalicylamido)ethyl-1-3'-dithiopropionate ("SASD"), N-maleimidobenzoyl-N-hydroxysuccinimidyl ester ("MBS"), m-maleimidobenzoylsulfosuccinimide ester ("sulfo-MBS"), N-succinimidyl-(4-iodoacetyl)aminobenzoate ("SIAB"), succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate("SMCC"), succinimidyl-4-(p-maleimidophenyl)butyrate ("SMPB"), sulfosuccinimidyl(4-iodoacetyl)aminobenzoate ("sulfo-SIAB"), sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate ("sulfo-SMCC"), sulfosuccinmidyl 4-(p-maleimidophenyl)butyrate ("sulfo-SMPB"), bromoacetyl-p-aminobenzoyl-N-hydroxy-succinimidyl ester, iodoacetyl-N-hydroxysuccinimidyl ester, and the like.

MIP may be conjugated to a carrier molecule accoding to the methodologies which have been used for conjugating other biologically active proteins ("bioactive proteins") to carriers. These methodologies, as described for example in U.S. Patent No. 4,493,795, are as follows:

(1) Coupling via α- or β-amino groups:

The carboxyl functional groups on a carrier may be activated with carbodiimides (especially water soluble carbondiimides (WSC) such as N-ethyl-N'-(3-dimethylaminopropylcarbodiimide), isoxazolium salts

(*e.g.* Woodwards Reagent K), 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), active ester-forming reagents (to yeild N-hyroxysuccinimide esters, 1-hydroxybenzotriazole esters, nitrophenyl esters, pentafluorophenyl esters, etc.), reagents yielding acid chorides (*e.g.*, PCl_5, but only for non-protein carriers), reagents yielding mixed anhydrides (*e.g.*, isobutyl-chloroformate, acetic anhydride, pivalic anhydride).

The free amino function of the bioactive protein (either α-amino function or ϵ-amino function of lysine) is then allowed to react with the activated carboxyl function of the carrier in an aqueous buffer (pH may be from about 6.5 to 9, optimally about 8) or in a mixed organic/aqueous buffer system (*e.g.*, DMF/waterm pH 8). For non-protein carriers, an organic solvent (*e.g.*, DMF) may be used. Especially useful techniques in this class are concurrent activation of the protein carrier with WSC and coupling with the bioactive protein in aqueous buffer or preparation of the p-NO_2-phenyl ester of a succinylated protein carrier followed by coupling with the bioactive agent in aqueous buffer.

The amino function(s) on the bioactive protein may be crosslinked with amino functions on the carrier molecule by reaction with gluteraldehyde in aqueous solution on mixed organic/aqueous solution (pH~7) at room temperature.

The amino function(s) on the bioactive protein may be crosslinked with amino functions on the carrier molecule by reaction with bifunctional crosslinking reagents such as dimethylsuberimidate, phenyldiisocyanate, phenyldiisothiocyanate, difluorodiitrobenzene, or cyanic chloride.

(2) <u>Coupling via α, β or γ carboxyl groups:</u>

The carboxyl functions on the bioactive protein will be activated by the techniques listed above for carboxyl activation. The activated carboxyl functions

will then be reacted with the amino functions on a suitable carrier molecule using the aqueous or mixed organic/aqueous buffer conditions described above.

(3) Coupling via thiol groups:

The –SH groups on the bioactive protein (incorporated as cysteinyl or homocysteinyl residues on or in the polypeptide chain) will be reacted with suitable carriers which have been modified by the incorporation of maleimide functions. The –SH function inserts specifically into the double bond of the maleimide function and yields a protein-carrier complex in which the carrier has retained a monomeric nature. The SH function may be incorporated into the bioactive protein by either incorporation of a Cys (or homo-Cys) residue in α-amino acid linkage, or by reaction of an amino function (α-amino or ϵ-amino of lysine) with cysteine thiolactone. Alternatively, the –SH function on the bioactive protein may be activated as the 2- or 4-thiopyridyldisulfide and bonded to the –SH groups on a suitable carrier. This sequence may also be reversed with the carrier –SH activated as the thiopyridyldisulfide.

(4) Coupling via aromatic rings:

The aromatic rings of Tyr and His may be crosslinked to the aromatic rings of Tyr and His residues of proteins by means of bis-diazotized aromatic compounds (*e.g.*, bis-diazotized-benzidine or bis-diazotized o-anisidide). This reaction is performed on an aqueous or mixed organic/aqueous solution of the bioactive protein and carrier.

Also included in the scope of such associations is the formation of a unitary protein by genetic engineering, resulting from the co-expression of genetic information for all or part of MIP and the carrier molecule as a single protein.

According to an exemplary treatment protocol, 2.5 mg of MIP or MIP analog of generally equivalent potency is administered intravenously to a 70 kg patient having distal ischemia, stroke, or other thromboembolic phenomena associated with abnormally elevated platelet count. The platelet count and function are monitored from seven to ten days after administration by analysis of blood samples taken at 4-hour intervals to evaluate MIP potency and clearance rates. At the end of the evaluation period, the dosage is adjusted as necessary to establish an improved platelet count or function, and the patient is again monitored once or twice weekly, as described. At the end of the period, the MIP dosage is again adjusted as necessary, with repetition of the described monitoring and evaluation procedure until the platelet count is substantially stabilized at a normal or near-normal level. The dosage required to obtain the desired stabilized platelet count comprises a therapeutic dosage according to the present invention. Indefinite daily administration of the therapeutic dosage may be necessary in order to maintain normal platelet levels during chronic thrombocytosis.

The practice of the invention is illustrated by the following non-limiting example.

Example

The ability of the MIPs to inhibit megakaryocyte colony formation was demonstrated by the following assay.

Megakaryocyte colonies were cloned in plasma clot cultures as previously described (Blood 61, 384-9 (1983)). The cell population cultured consisted of either unseparated high density marrow mononuclear cells (MNC), or MNC depleted of adherent monocyte-macrophages and T lymphocytes using methods previously reported (J. Immunol. 139, 2915-2925 (1987)). To

estimate basal growth conditions in marrow, the cultures contained no exogenous source of growth factors. To provide such essential growth factors, all cultures were supplemented with normal human AB serum (30% v/v) derived from the platelet-poor plasma of a single donor. Various amounts of pure recombinant murine MIP-1α and -1β, and pure recombinant MIP-2α and -2β, were added to the unseparated marrow MNC.

Megakaryocyte colonies were enumerated by indirect immunofluorescence assay utilizing a rabbit anti-human platelet glycoprotein antiserum as a megakaryocyte probe (ibid.). The antiserum used was highly specific for recognition of platelet glycoproteins. It does not recognize monocytes. A cluster of three or more intensely fluorescent cells was counted as one colony. The aggregate results of three such experiments are shown in Figure 1 (MIP-1α and -1β) and Figure (MIP-2α and -2β).

All four MIPs displayed an essentially equal ability to inhibit megakaryocyte colony formation in plasma clots, causing ~35% and ~50% inhibition at concentrations of 50 ng/ml and 500 ng/ml, respectively.

All references cited with respect to synthetic, preparative and analytical procedures are incorporated herein by reference.

The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

SEQUENCE LISTING

(1) **GENERAL INFORMATION:**

(i) **APPLICANT(S):** Gewirtz, Alan M.

(ii) **TITLE OF INVENTION:** Suppression of megakaryocytopoiesis by macrophage inflammatory proteins

(iii) **NUMBER OF SEQUENCES:** 4

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(v) **COMPUTER READABLE FORM:**

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(viii) **ATTORNEY/AGENT INFORMATION:**

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(C) **REFERENCE/DOCKET NUMBER:** 3957-9 PC

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(C) **TELEX:** None

(2) **INFORMATION FOR SEQ ID NO:1:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 92 amino acids

(B) **TYPE:** amino acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:1:**

Met	Gln	Val	Ser	Thr	Ala	Ala	Leu	Ala	Val	Leu	Leu	Cys	Thr	Met
				5					10					15
Ala	Leu	Cys	Asn	Gln	Phe	Ser	Ala	Ser	Leu	Ala	Ala	Asp	Thr	Pro
				20					25					30
Thr	Ala	Cys	Cys	Phe	Ser	Tyr	Thr	Ser	Arg	Gln	Ile	Pro	Gln	Asn
				35					40					45
Phe	Ile	Ala	Asp	Tyr	Phe	Glu	Thr	Ser	Ser	Gln	Cys	Ser	Lys	Pro
				50					55					60
Gly	Val	Ile	Phe	Leu	Thr	Lys	Arg	Ser	Arg	Gln	Val	Cys	Ala	Asp
				65					70					75
Pro	Ser	Glu	Glu	Trp	Val	Gln	Lys	Tyr	Val	Ser	Asp	Leu	Glu	Leu
				80					85					90
Ser	Ala													
	92													

(2) **INFORMATION FOR SEQ ID NO:2:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 92 amino acids

(B) **TYPE:** amino acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:2:**

Met	Lys	Leu	Cys	Val	Thr	Val	Leu	Ser	Leu	Leu	Met	Leu	Val	Ala
				5					10					15
Ala	Phe	Cys	Ser	Leu	Ala	Leu	Ser	Ala	Pro	Met	Gly	Ser	Asp	Pro
				20					25					30
Pro	Thr	Ala	Cys	Cys	Phe	Ser	Tyr	Thr	Ala	Arg	Lys	Leu	Pro	Arg
				35					40					45
Asn	Phe	Val	Val	Asp	Tyr	Tyr	Glu	Thr	Ser	Ser	Leu	Cys	Ser	Gln
				50					55					60
Pro	Ala	Val	Val	Phe	Gln	Thr	Lys	Arg	Ser	Lys	Gln	Val	Cys	Ala
				65					70					75
Asp	Pro	Ser	Glu	Ser	Trp	Val	Gln	Glu	Tyr	Val	Tyr	Asp	Leu	Glu
				80					85					90
Leu	Asn													
	92													

(2) **INFORMATION FOR SEQ ID NO:3:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 73 amino acids

(B) **TYPE:** amino acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:3:**

Ala	Pro	Leu	Ala	Thr	Glu	Leu	Arg	Cys	Gln	Cys	Leu	Gln	Thr	Leu
				5					10					15
Gln	Gly	Ile	His	Leu	Lys	Asn	Ile	Gln	Ser	Val	Lys	Val	Lys	Ser
				20					25					30
Pro	Gly	Pro	His	Cys	Ala	Gln	Thr	Glu	Val	Ile	Ala	Thr	Leu	Lys
				35					40					45
Asn	Gly	Gln	Lys	Ala	Cys	Leu	Asn	Pro	Ala	Ser	Pro	Met	Val	Lys
				50					55					60
Lys	Ile	Ile	Glu	Lys	Met	Leu	Lys	Asn	Gly	Lys	Ser	Asn		
				65					70			73		

(2) **INFORMATION FOR SEQ ID NO:4:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 73 amino acids

(B) **TYPE:** amino acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:4:**

Ala	Ser	Val	Val	Thr	Glu	Leu	Arg	Cys	Gln	Cys	Leu	Gln	Thr	Leu
				5					10					15
Gln	Gly	Ile	His	Leu	Lys	Asn	Ile	Gln	Ser	Val	Asn	Val	Arg	Ser
				20					25					30
Pro	Gly	Pro	His	Cys	Ala	Gln	Thr	Glu	Val	Ile	Ala	Thr	Leu	Lys
				35					40					45
Asn	Gly	Lys	Lys	Ala	Cys	Leu	Asn	Pro	Ala	Ser	Pro	Met	Val	Gln
				50					55					60
Lys	Ile	Ile	Glu	Lys	Ile	Leu	Asn	Lys	Gly	Ser	Thr	Asn		
				65					70			73		

CLAIMS

1. A method for reducing the number of circulating platelets in the bloodstream of a mammal comprising administering to the mammal an amount of macrophage inflammatory protein-1 and/or macrophage inflammatory protein-2 effective to induce such a reduction.

2. A method according to claim 1 wherein the amount of macrophage inflammatory protein administered is sufficient to reduce the number of circulating platelets by at least about 10%.

3. A method according to claim 1 for treatment of thrombocytosis comprising administering to a human being a daily dosage of macrophage inflammatory protein of from about 0.1 to about 500 mg.

4. A method according to claim 3 wherein the daily dosage is from about 0.5 to about 50 mg.

5. A method according to claim 1 wherein the protein is macrophage inflammatory protein-1α.

6. A method according to claim 1 wherein the protein is macrophage inflammatory protein-1β.

7. A method according to claim 1 wherein the protein is macrophage inflammatory protein-2α.

8. A method according to claim 1 wherein the protein is macrophage inflammatory protein-2β.

9. A method for reducing the number of circulating platelets in the bloodstream of a mammal comprising administering to the mammal an amount of a macrophage inflammatory protein-1 analog or macrophage inflammato-

ry protein-2 analog effective to induce such a reduction.

10. A method according to claim 9 wherein the amount of macrophage inflammatory protein analog administered is sufficient to reduce the number of circulating platelets by at least about 10%.

11. A method according to claim 9 wherein the analog has at least 80% sequence homology any of human macrophage inflammatory proteins-1α, -1β, -2α or -2β.

12. A method according to claim 11 wherein the analog has at least 90% sequence homology with the human macrophage inflammatory protein.

13. A method according to claim 9 for treatment of thrombocytosis comprising administering to a human being a daily dosage of macrophage inflammatory protein analog of from about 0.1 to about 500 mg.

14. A method according to claim 13 wherein the daily dosage is from about 0.5 to about 50 mg.

15. A method for reducing the number of circulating platelets in the bloodstream of a mammal comprising administering to the mammal a platelet number-reducing effective amount of a conjugate comprising macrophage inflammatory protein-1, macrophage inflammatory protein-2, or analog of either operatively linked to a pharmaceutically acceptable carrier molecule.

16. A method according to claim 15 wherein the carrier molecule comprises a protein.

17. A method according to claim 15 wherein the conjugate comprises a protein operatively linked to macrophage inflammatory protein-1a.

18. A method according to claim 15 wherein the conjugate comprises a protein operatively linked to macrophage inflammatory protein-1b.

19. A method according to claim 15 wherein the conjugate comprises a protein operatively linked to macrophage inflammatory protein-2a.

20. A method according to claim 15 wherein the conjugate comprises a protein operatively linked to macrophage inflammatory protein-1b.

21. Use of macrophage inflammatory protein-1 and/or macrophage inflammatory protein-2 for the manufacture of a medicament for reducing the number of circulating platelets in the bloodstream of a mammal.

22. Use according to claim 21 wherein the amount of macrophage inflammatory protein is sufficient to reduce the number of circulating platelets in said mammal by at least about 10%.

23. Use according to claim 21 for treatment of thrombocytosis.

24. Use according to claim 21 wherein the protein is macrophage inflammatory protein-1α.

25. Use according to claim 21 wherein the protein is macrophage inflammatory protein-1β.

26. Use according to claim 21 wherein the protein is macrophage inflammatory protein-2α.

27. Use according to claim 21 wherein the protein is macrophage inflammatory protein-2β.

28. Use of a macrophage inflammatory protein-1 analog or macrophage inflammatory protein-2 analog for the manufacture of a medicament for reducing the number of circulating platelets in the bloodstream of a mammal.

29. Use according to claim 28 wherein the amount of macrophage inflammatory protein analog administered is sufficient to reduce the number of circulating platelets by at least about 10%.

30. Use according to claim 28 wherein the analog has at least 80% sequence homology with any of human macrophage inflammatory proteins-1α, -1β, -2α or -2β.

31. Use according to claim 30 wherein the analog has at least 90% sequence homology with said macrophage inflammatory protein.

32. Use according to claim 28 for treatment of thrombocytosis.

33. A conjugate comprising macrophage inflammatory protein-1, macrophage inflammatory protein-2, or analog of either, operatively linked to a pharmaceutically acceptable carrier molecule.

34. Use conjugate according to claim 33 wherein the carrier molecule comprises a protein.

35. A conjugate according to claim 34 comprising macrophage inflammatory protein-1a operatively linked to a protein.

36. A conjugate according to claim 34 comprising macrophage inflammatory protein-1b operatively linked to a protein.

37. A conjugate according to claim 34 comprising macrophage inflammatory protein-2a operatively linked to a protein.

38. A conjugate according to claim 34 comprising macrophage inflammatory protein-2b operatively linked to a protein.

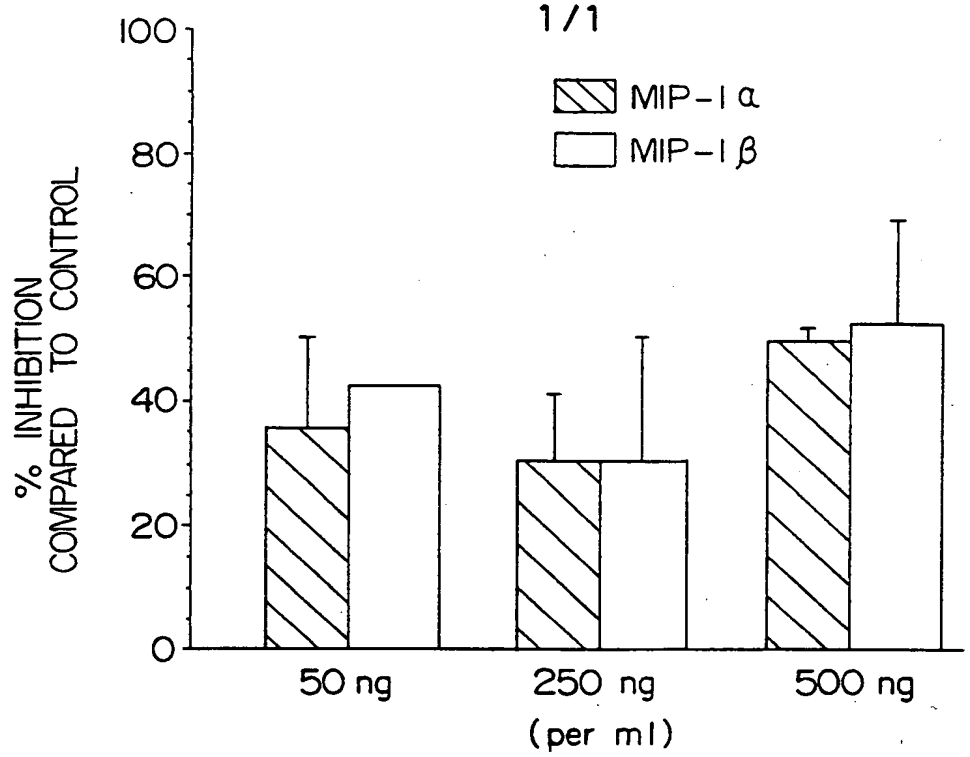
100
80
60
40
20
0
% INHIBITION COMPARED TO CONTROL
MIP-1α
MIP-1β
50 ng
250 ng
500 ng
(per ml)

FIG. 1

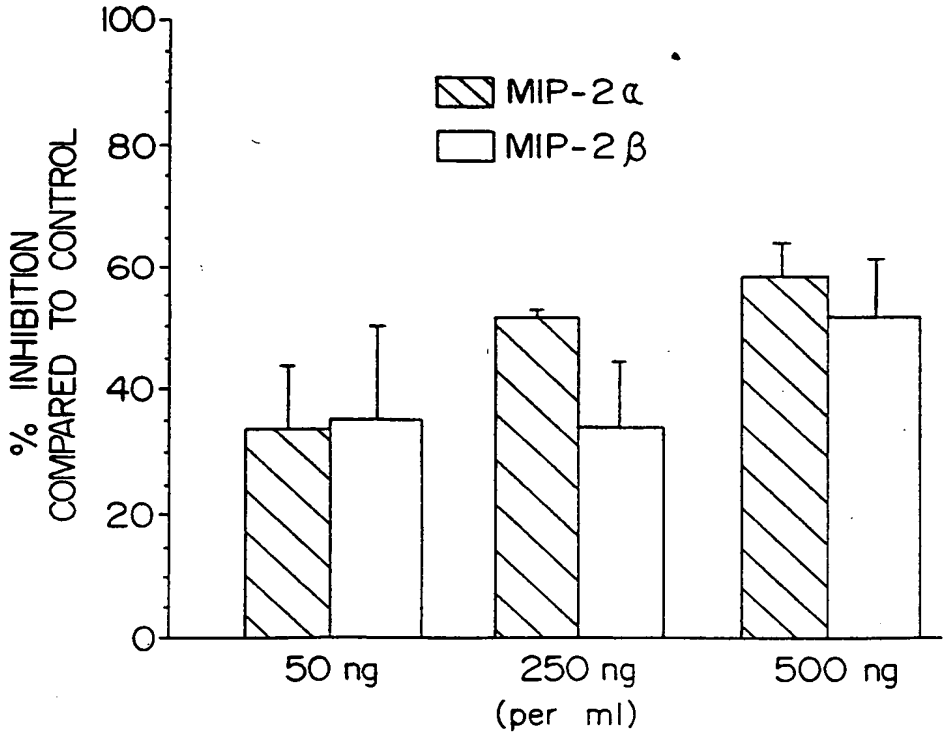
100
80
60
40
20
0
% INHIBITION COMPARED TO CONTROL
MIP-2α
MIP-2β
50 ng
250 ng
500 ng
(per ml)

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09671

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/02
US CL :514/12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG:BIOSIS PREVIEWS; MEDLINE; WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	BLOOD, Volume 76, No. 6, issued 15 September 1990, H. E. Broxmeyer et al, "Enhancing and Suppressing Effects of Recombinant Murine Macrophage Inflammatory Proteins on Colony Formation In Vitro by Bone Marrow Myeloid Progenitor Cells", pages 1110-1116, especially page 1115.	1-14, 21-32 ——— 15-20, 33-38
X — Y	NATURE, Volume 344, issued 29 March 1990, G. J. Graham et al, "Identification and Characterizarion of an Inhibitor of Haemopoietic Stem Cell Proliferation", pages 442-444, especially page 444.	1-14, 21-32 ——— 15-20, 33-38
Y	US, A, 4,493,795 (NESTOR, JR. ET AL) 15 JANUARY 1985, see entire document.	15-20, 33-38
Y	US, A, 4,671,958 (RODWELL ET AL) 09 JUNE 1987, see entire document.	15-20, 33-38

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 28 January 1993	Date of mailing of the international search report 10 FEB 1993
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer CHOON P. KOH Telephone No. (703) 308-0196

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